# Basic Investigation

## Preventive Administration of Juanbi Capsules (蠲痹胶囊) for Knee Osteoarthritis: Effects on Serum MMP-2 and MMP-9 levels and Cartilage Repair

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**Objective:** To explore the mechanism of action of Juanbi Capsules (蠲痹胶囊), a Chinese medicine for invigorating the kidney and replenishing *qi*, in preventing osteoarthritis of the knee in rabbits.
**Methods:** Seventy-two 4-month-old, Japanese long-eared white rabbits were randomly divided into 6 groups: control (group A), model (group B), Chinese drug; high-dose (group C), Chinese drug; mid-dose (group D), Chinese drug; low-dose (group E), and drug control (group F). With the exception of the rabbits in group A, each rabbit was subjected to plaster cast fixation for 6 weeks to induce osteoarthritis. In addition, rabbits were administrated with an intragastric injection of the Chinese drug (groups C, D and E) or an aminoglucose hydrochloride capsule (group F) for 4 weeks. Blood was drawn from the central ear artery for serum MMP-2 and MMP-9 concentrations, and the knee joint cartilage was harvested for gross observation and light microscopy.
**Results:** There were significant differences in serum MMP-2 and MMP-9 concentrations between group B and groups C, D and E ($P<0.05$), with no significant differences between groups D and F. Histological results showed various changes in tissue staining with treatment, with osteophyte and bone cyst formation, and superficial erosion in the articular surface of the cartilage; in some cases, the defect reached the mid-layer of the cartilage, and these changes were lower than those in the model group.
**Conclusion:** Juanbi Capsules assist in preventing osteoarthritis in the rabbit, possibly by decreasing serum MMP-2 and MMP-9 levels.

***Keywords:*** *Invigorating kidney and replenishing qi; osteoarthritis, prevention, MMP-2, MMP-9*

Osteoarthritis (OA), or hyperplastic arthritis, is pathologically characterized by progressive articular cartilage degeneration, articular margin proliferation and reactive changes to the sub-chondral substance. OA is one of the most prevalent types of osteoarthropathy, and the mortality associated with OA increases with age. At present, there is no effective method for reversing or preventing its onset; the only treatment available is to alleviate symptoms and delay the progression of the disease. Therefore, there is increasing pressure on researchers to identify mechanisms to prevent this disease. In Traditional Chinese Medicine (TCM), disease prevention plays an important guiding role in the clinical aspects of patient care. Thus, investigating prevention and early treatment of OA are essential.[1]

Our group posits that the main pathogenesis of OA stems from deficiency in both the liver and the kidney, a deficiency in *qi* and blood, and weak muscles and joints. Clinically, reinforcing and replenishing the kidney with the Chinese compound, Juanbi Capsules, has been used in the prevention and treatment of early OA, with obvious therapeutic effects. The following experiment was carried out in order to reveal this mechanism of action in preventing early OA.

## MATERIALS AND METHODS

### Experimental Animals

Seventy-two healthy male Japanese long-eared rabbits, aged 4 months, weighing 1.8–3.5 kg, were supplied by the Center for Experimental Animals, Xi'an Communication University, Shaanxi Province, China, license No. SCXK (Shan) 2008-008. All rabbits were maintained for one week at room temperature before the experiment, with free access to food and water.

### Experimental Drugs and Reagents

Juanbi Capsules (蠲痹胶囊) were composed of: 15 parts each of Shu Di (Radix Rehmanniae Preparata), Sheng Huang Qi (Radix Astragali), Yin Yang Huo (Herba Epimedii), Gu Sui Bu (Rhizoma Drynariae); 10 parts each of Rou Cong Rong (Herba Cistanchis) and Niu Xi

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(Radix Achyranthis Bidentatae), and 6 parts of Gan Cao (Radix Glycyrrhizae). These compounds were supplied by the TCM Pharmacy of the Affiliated Hospital of Shaanxi TCM College, Xianyang, China. The compounds were added with 10-fold of water, decocted and twice extracted. The extract solutions were combined and concentrated by reducing pressure into a relative density of 1.1 (50°C), combined with alcohol for sedimentation, to a final concentration of 60% alcohol content. The supernatant was taken, the alcohol was recovered, and the solution concentrated and dried with reducing pressure, then ground into fine powder. Just before use, the TCM powder of a corresponding dose was dissolved in water for administration. Aminoglucose hydrochloride capsules were purchased from Zhejiang Chengyi Pharmaceutical Co. Ltd, Dongtou, Zhejiang, China (Production lot No: 20090503, State drug No: H20060748). Matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9) kits were supplied by R&D System Co. (Minneapolis, MN, USA).

**Experimental Instruments**

Experiments were carried out using a HB-2 Olympus microscope, CBQ-II ultramicrotome, a DL-8R high-speed, low temperature refrigerated centrifuge, and an ELX808IU microplate reader (FCC Compliance, USA).

**Modeling and Group Assignment**

Seventy-two rabbits were randomly divided into the following groups: control (group A), model (group B), TCM high-dose (group C), TCM mid-dose (group D), TCM low-dose (group E), drug control (group F), with 12 rabbits in each group. Except for the rabbits in group A, the animal model was created using plaster cast fixation for 6 weeks to induce osteoarthritis in the rabbits, using the reported method.[2] At the same time, the rabbits in the treatment groups were intragastrically administrated with different doses of Juanbi Capsules (groups B, C, D, and E) and aminoglucose hydrochloride capsules (group F) for 4 weeks. After 6 weeks, the rabbits were assessed for histological and gross changes.

**Drug Administration**

Drugs were administrated by intragastric injection for 4 weeks from the first day of modeling. For groups C, D and E, Juanbi Capsules of 20-, 10- and 6-fold of the clinical adult dose, respectively (corresponding to crude drug amounts of 96, 46 and 27.6 g, respectively) were administrated twice daily, in the morning and evening. At the same time points, aminoglucose hydrochloride capsules, corresponding to a 5-fold adult dose, were administered by intragastric injection to group F, and an equal volume of saline (2 mL/d) to group B. Rabbits in group A received no treatment.

**Observation of General State**

The general state of the animal, including body weight, level of activity, hair luster, urine and stool samples, and the gross form of the articular cartilage of knee joint were observed.

**Histomorphological Observations**

Histological changes of articular cartilage were observed using microscopy**.**

**Determination of Serum MMP-2 and MMP-9 Levels**

After modeling for 6 weeks, the rabbits were anesthetized with an intraperitoneal injection of 2% phenobarbital sodium (30 mg/kg). Blood (2 mL) was then drawn from central artery of the ear, and centrifuged at 4°C, 3000 r/min, for 5 min. The serum was taken and kept at -20°C for detection of MMP-2, MMP-9 levels with ELISA method, according to the manufacturer's instructions.

**Statistical Analysis**

The data were expressed as mean ± standard deviation ($\bar{x} \pm s$), and analysis of variance (ANOVA) was used for comparison between groups.

## RESULTS

**Observation of Animal General State and Condition of Knee Joint Cartilage**

The rabbits in group A showed no abnormal changes in activity level or hair color, but were more sensitive to external stimulation and the stable fixation of the plaster support for the knee joint. The rabbits in the group B had withered hair color with no luster, reduced spontaneous activity, and decreased sensitivity to external stimulation during fixation of the plaster support. The rabbits in groups C, D, E, and F, showed more luster in their coats and increased spontaneous activity, as compared with group B. They also showed increased sensitivity to external stimulation with plaster fixation.

**Histological Changes of Cartilage of Knee Joint in the Groups**

After modeling for 6 weeks, the rabbits were euthanized and the left knee joint harvested. Using microscopy, H&E and collagen staining showed an incomplete cartilage membrane for the rabbits in group B. Specifically, the surface fissure reached the calcification belt, the structure was completely destroyed, and the tissue showed irregular cellular form, with less cells in the superficial layer and an irregular arrangement of collagen fibers. In group A, a smooth cartilage membrane was observed without a surface fissure. IN addition, the tissue showed regular arrangement of cartilage cells, with a horizontal arrangement in the upper layer and a perpendicular arrangement in the lower layer; and a normal number of circular cells. In the groups C, D, E and F, the structures were destroyed to varying degrees, but milder than those in the group B.

**Effects of Juanbi Capsules on Serum MMP-2 and MMP-9**

Overall, there were statistically significant differences in the serum MMP-2 and MMP-9 levels for groups C, D, E, F as compared with group B (all $P<0.05$). Similarly, significant differences were observed for groups C and E as compared with group F ($P<0.05$). No significant differences between were observed between group D and group F ($P>0.05$). These results are listed in Table 1.

**Table 1.** Effects of Juanbi Capsules on serum MMP-2 and MMP-9 contents

| Group | *n* | MMP-2 (ug/L) | MMP-9 (ug/L) |
|---|---|---|---|
| A | 12 | 0.250±0.001※ | 0.509±0.004※ |
| B | 12 | 23.854±4.164▲ | 57.367±1.294▲ |
| C | 12 | 9.250±3.823* | 20.904±3.822* |
| D | 12 | 13.376±0.673* | 26.613±4.066* |
| E | 12 | 22.704±0.497* | 50.038±1.871* |
| F | 12 | 13.300±0.964★□ | 25.809±2.942★□ |

Notes: Group A compared with group B, ※$P<0.05$; Group B compared with groups C, D, E and F, ▲$P<0.05$; Group F compared with groups C and E, ★$P<0.05$; Comparison among groups C, D, E, *$P<0.05$; Group F compared with groups C, D, □$P>0.05$.

## DISCUSSION

Osteoarthritis (OA) is a disease characterized by retrograde degeneration of the joint cartilage, with subcartilagenous proliferation. In 1995, the International OA Meeting put forward that OA was induced by an imbalance of the normal coupling between degradation and synthesis in cartilage cells, extracellular matrix and subcartilagenous bone, under the common action of mechanical and biological factors.[1]

The most recent study[3] indicates that OA is linked to biomechanical factors, the imbalance between synthesis and degradation of the cartilaginous matrix, an abnormal differentiation of cartilaginous cells in the joint, aging of the joint cartilage, and abnormal activity of subcartilagenous bone, among others. Kim et al.[4] showed that the in early the stages of OA are characterized by changes in the form and metabolism of cartilagenous cells, the distribution and structure of proteoglycans, and the metabolism of collagen fibers in cartilage before destruction of collagen fiber network structure. This was supported by another recent paper showing that one of the most important causes of cartilage degeneration in OA is an imbalance in the synthesis and degradation of the extracellular matrix in cartilage.[5]

Recent work has shown the potential role of matrix metalloproteinases (MMPs) in this disease. MMPs are a group of $Zn^{2+}$ ion-dependent proteolytic enzymes, involved in the degradation of the extracellular matrix through hydrolysis.[6] MMPs are synthesized and secreted mainly by fibroblasts, endothelial cells, macrophages, and neutrophilic granulocytes, among others. MMPs can be divided into 4 categories according to their different substrates: interstitial collagenases, W-type collagenases (also called as gelatinases, i.e., MMP-2 and MMP-9), stromatolysis enzymes and membrane type-matrix metalloproteinases, which mainly includes collagenases (MMP 1, 8 and 13), matrix lysins (MMP 3, 7 and 10), and gelatinases (MMP2 and 9). Collagenases are the main enzymes responsible for collagen degradation, and MMP 13 has the strongest action. At present, it is a commonly held belief that MMP sub-families show direct specificity in the degradation of collagens, leading to destruction of the collage network. This, in turn, causes cartilage cells embedded in the extracellular matrix to become exposed to various inflammatory factors that, in addition to the action of mechanical loading, leads to cartilage degradation.[7]

Currently, the pathogenic mechanism of OA is not completely clear. From a pathological angle, it is hypothesized that cartilage degeneration in OA patients is due to a lesion induced by an imbalance between extracellular matrix degradation and synthesis in cartilage.[8] Because MMPs show distinct degradation abilities on the various components of the matrix, it is likely that MMPs have different roles during the discrete stages of cartilage degeneration.[9] Due to the close relationship between OA and MMPs, MMPs have been become the new target for effective OA treatment .[10,11]

TCM has always focused on disease prevention. OA is included in the category of “rheumatism involving the bone”, and “pain in the waist and lower extremities”. OA starts from a deficiency in the origin and an excess in the superficial region – for instance, OA induced by a deficiency in liver-*yin* and kidney-*yang*. The origin concentrates on stagnation of pathogenic wind-cold-dampness in channels and collaterals, tonifying the liver and the kidney, strengthening the muscles and bones, invigorating *qi* and blood; treatment of the superficiality includes methods for expelling the wind, clearing away cold, removing dampness, activating blood circulation, dredging collaterals and analgesia, with good results. Therefore, drugs for tonifying the liver and kidney have important effects in the early prevention and treatment of OA. Our previous study indicated that the Chinese drugs for invigorating the kidney and replenishing *qi* prevent OA possibly by decreasing serum TNF-α, IL-1 and IL-6 levels.[2]

In summary, this study shows that Juanbi Capsules may reduce degradation of cartilage matrix, and prevent cartilage degeneration in OA by decreasing serum MMP-2 and MMP-9 levels. This could ultimately delay degeneration of the joint and improve joint function. Our results therefore provide a reliable theoretical basis for the early treatment of osteoarthritis with TCM.

## REFERENCES

1. Goldring SR, Goldring M B. The role of cytokines in

cartilage matrix degeneration in osteoarthritis. J Clinical Orthopaedics and Related Research 2004: 27-36.
2. Yuan PW, Liu DY, Chu XD, Hao YQ, Zhu C, Qu Q. Effects of preventive administration of Juanbi Capsules on TNF-α, IL-1 And IL-6 contents of joint fluid in the rabbit with knee osteoarthritis. Journal of Traditional Chinese Medicine (Chin) 2010; 30: 254-258.
3. Wei XC. Articular Carlitage(Chin), 1st edition. Science Publishing House, September 2007.
4. Kim SJ, Kim YH. The modulation of integrin expression by the extracellular matrix in articular chondrocytes. Yonsei Med J 2003; 44: 493
5. Fietz S, Einspanier R, Hoppner S, Hertsch B, Bondzio A. Determination of MMP-2 and -9 activities in synovial fluid of horses with osteoarthritic and arthritic joint diseases using gelatin zymography and immunocapture activity assays. Equine Vet J 2008 May; 40: 266-271.
6. Kossakowska AE, Edwards DR, Lee SS. Altered balance between matrix matalloproteineses and their inhibitors in experimental biliary fibrosis. Am J.Pathal 1998; 153: 1895.
7. Lawyer T, Wingerter S, Tucci M, Benghuzzi H. Cellular effects of catabolic inflammatory cytokines on chondrocytes-biomed 2011. Biomed Sci Instrum 2011; 47: 252-257.
8. Dumond H, Presle N, Pottie P, et al. Sites peeifieehangsin gene expression and cartilage metabolism during early experimental osteoarthritis. Osteoarthritis Cartilage 2004; 12: 284-295.
9. Ren ZW, Yu YL, Yang FJ, Qiao Jian, Jian JY, XIAOBG, et al. Effects of IL-1β on MMP-1/-13 mRNA expressions and NO in articular chondrocytes of the rabbit. Fudan Journal (Medical Edition, Chin.) 2008; 35: 167-170.
10. Bao F, Sun H, Wu ZH, Wang DH, Zhang YX. Effect of acupuncture on expression of matrix metalloproteinase and tissue inhibitor in cartilage of rats with knee osteoarthritis. Chin Acup-Mox (Chin) 2011; 31: 241-246.
11. Kim KS, Choi HM, Lee YA, Choi IA, Lee SH, Hong SJ, et al. Yoo MC. Expression levels and association of gelatinases MMP-2 and MMP-9 and collagenases MMP-1 and MMP-13 with VEGF in synovial fluid of patients with arthritis. Rheumatol Int 2011; 31: 543-547.

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